Detection of katG Ser315Thr substitution in respiratory specimens from patients with isoniazid-resistant Mycobacterium tuberculosis using PCR-RFLP

Eric Tung-Yiu Leung, 1 Kai-Man Kam, 2 Agatha Chiu, 2 Pak-Leung Ho, 1, 3 Wing-Hong Seto, 1 Kwok-Yung Yuen 1, 4 and Wing-Cheong Yam 1

1 Department of Microbiology, The University of Hong Kong, University Pathology Building, Queen Mary Hospital, Pokfulam Road, Hong Kong SAR, China
2 Tuberculosis Reference Laboratory and Public Health Laboratory, Department of Health, Hong Kong SAR, China
3, 4 Centre of Infection 3 and HKU-Pasteur Research Centre 4 , Faculty of Medicine, The University of Hong Kong, Hong Kong SAR, China

Mutations in the katG locus of catalase peroxidase in Mycobacterium tuberculosis (MTB) account for major isoniazid (INH) resistance. In the South China region, a collection of 906 respiratory specimens and 142 MTB isolates was used to evaluate the sensitivity and specificity of a PCR-RFLP method for the detection of INH resistance-associated mutations. Except for four catalase-negative MTB isolates, katG PCR for a 620-bp amplicon was successful for all purified MTB isolates. For respiratory specimens, diagnostic sensitivity and specificity of katG PCR was 85 and 100 %.

Subsequent RFLP of the katG amplicons by MspI digestion identified that 51 % of INH-resistant MTB were associated with the Thr315 phenotype, and that codon 463 was a polymorphic site with no linkage to INH resistance. The Arg463 wild-type MTB isolates predominant in the Western world were replaced by isolates carrying Leu463 in the South China region. RFLP patterns of katG amplicons from respiratory specimens were identical to those of the corresponding MTB cultured colonies. This method has potential application for rapid diagnosis of INH resistance due to katG Ser315Thr mutation.

INTRODUCTION

Tuberculosis remains a global threat to public health. The problem is further complicated by the emergence of multi-drug-resistant tuberculosis as a consequence of the widespread use and incautious administration of antibiotics (WHO, 2000). Rapid diagnosis and appropriate chemotherapy become the first priorities in controlling growing epidemics. Nowadays, isoniazid (INH), ethambutol, rifampicin, pyrazinamide and streptomycin are important components of first-line anti-tubercular regimes. INH has a much more complex resistance mechanism than the other four compounds. Several studies have revealed different putative molecular targets of INH, including catalase peroxidase (katG) (van Soolingen et al., 2000), enoyl-acyl reductase (inhA) (Basso et al., 1998), alkyl-hydroperoxide reductase (ahpC) (Sherman et al., 1996), β-ketoacyl-acyl carrier protein synthase (kasA) (Mdluli et al., 1998) and NADH dehydrogenase (ndh) (Lee et al., 2001). However, a predominant mutation in the katG locus, Ser315Thr, accounts for more than 50 % of INH-resistance phenotypes, according to earlier reports (Musser et al., 1996; van Soolingen et al., 2000). Mycobacterial strains lacking the entire katG gene would also exhibit an INH-resistant phenotype (Heym et al., 1995).

In this study, we developed a simple PCR-RFLP method for direct detection of katG Ser315Thr-associated INH-resistant Mycobacterium tuberculosis (MTB) in clinical isolates and respiratory specimens. A total of 906 respiratory specimens and 142 MTB isolates were used to evaluate the specificity and sensitivity of this assay. Results were compared with commercial and in-house PCR assays for MTB, anti-mycobacterial susceptibility testing and DNA sequencing.
**METHODS**

**Specimens and isolates.** Between December 1999 and February 2002, 906 respiratory specimens (828 expectorated sputum and 78 bronchoalveolar lavage samples) collected from patients suffering from chest symptoms and/or chest radiographic infiltrates of undetermined origin included 675 patients from Queen Mary Hospital, 214 out-patients of Polyclinics of the Department of Health in Hong Kong. An additional 142 clinical isolates of MTB were collected from three major cities in the Pearl River delta of the South China region of China: Hong Kong (96 isolates), Macau (27 isolates) and Guangzhou (19 isolates). Specimens were processed for direct smear followed by concentration for acid-fast bacilli (AFB) culture as described previously (Nolte & Metchock, 1995). Cultures positive for AFB were identified using the AccuProbe hybridization assay (Gen-Probe) and culture and subsequent PCR assays. Cultures positive for AFB were processed for direct smear followed by concentration for acid-fast bacilli (AFB) culture as described previously (Nolte & Metchock, 1995). Isolates were randomly selected MTB isolates (10 INH-susceptible and 10 INH-resistant) were sequenced using BigDye technology and an ABI 377 Genetic Analyzer (Applied Biosystems).

**RESULTS AND DISCUSSION**

Among 906 respiratory specimens shown in Table 1, 233 were culture-positive for MTB and 35 were culture-positive for *Mycobacterium* sp. other than *tuberculosis* (MOTT), while the remaining 638 were culture-negative for *Mycobacterium*. Of 187 samples that were AFB smear-positive and culture-positive for MTB, 161 (86 %) were positive for *katG* PCR, whereas *IS6110* PCR and Roche PCR provided 100 % sensitivity for direct detection of MTB in these specimens. This result is concordant with an experiment on analytical sensitivity that showed that *IS6110* PCR was 10 times more sensitive than the *katG* PCR (Fig. 1). End-point detection of MTB for *IS6110* PCR and *katG* PCR was found to be 15 % and 15 c.f.u., respectively. Using a simple column-elution concentrator, all 187 AFB smear-positive specimens and 11/46 (24 %) AFB smear-negative specimens were positive for *katG* PCR. A total of 233 specimens were subsequently confirmed to be culture-positive for MTB, all of which were found to be catalase-positive. A specificity of 100 % was exhibited for the three PCR assays on 906 respiratory specimens with diagnostic sensitivity of 92 % for *IS6110* PCR, 86 % for Roche PCR and 85 % for *katG* PCR. The *katG* PCR is highly specific for MTB, in that none of the specimens with MOTT or those that were culture-positive for other respiratory bacterial pathogens showed a positive result. By Roche PCR, 4-8 % specimens overall were detected to contain PCR inhibitors (data not shown).

The *katG* amplicons were further analysed by RFLP using *MspI* digestion (Table 2) and four distinct RFLP patterns were generated (Fig. 2). All RFLP patterns of *katG* amplicons from the 198 respiratory specimens were identical to those of the corresponding MTB isolates, indicating that *katG* PCR provides reliable detection in clinical specimens. Four catalase-negative MTB isolates were *katG* PCR-negative and confirmed INH-resistant by susceptibility testing. These isolates confer INH resistance by partial or complete deletion of *katG*, accounting for the negative *katG* PCR result (Heym et al., 1995). Among the 375 MTB isolates (233 isolates from respiratory specimens and 142 isolates from purified culture), the proportional method identified 273 INH-susceptible and 102 INH-resistant MTB isolates. Of the 102 INH-resistant isolates (Hong Kong, 71 isolates; Macau, 19; Guangzhou, 12), 52 (51 %) isolates exhibited phenotypeThr315 (RFLP patterns C and D). The remaining 50 (49 %) resistant isolates showed phenotypeSer315 (RFLP patterns A and B). There was no documentation of an outbreak during the study period, and all RFLP patterns were distributed randomly among isolates from the three cities (data not shown). Thr315 is 100 % specific for INH resistance, since no susceptible isolates exhibited Thr315. Automated DNA sequencing of the 620-bp *katG* amplicons from 10 randomly
selected INH-susceptible isolates (four isolates of RFLP pattern A and six isolates of RFLP pattern B) and 10 INH-resistant isolates (two, one, three and four isolates, respectively, of RFLP patterns A–D) verified 100 % sequence accuracy of the point mutations detected by PCR-RFLP. DNA sequencing also revealed no mutation other than Ser/Thr315 and Arg/Leu463 within the 620-bp $katG$ amplicons for the 20 isolates. Arg463 and Leu463 were identified in 20 % ($20/102$) and 80 % ($82/102$), respectively, of the resistant isolates. Codon 463 is a polymorphic site that does not contribute to INH resistance (van Doorn et al., 2001). In this study, Leu463 was the predominant wild-type MTB isolate in the South China region, replacing the major Arg463 wild-type found in the Western world (Cockerill et al., 1995).

Previous findings showed rapid detection of INH resistance
in purified MTB colonies using a real-time PCR assay (Garcia de Viedma et al., 2002) or allele-specific PCR assay (Mokrousov et al., 2002); this is the first report of direct detection of INH resistance-associated mutations of MTB in clinical specimens with highly specific results. Our protocol requires a simple laboratory set-up suitable for a diagnostic microbiology service and the turnaround time can be shortened from 8–10 weeks to 3 days. In routine practice, respiratory specimens positive for IS6110 or Roche PCR should be subjected to subsequent katG PCR. For katG PCR-positive samples, RFLP should be applied to identify the Ser315Thr substitution. For katG PCR-negative samples, routine mycobacterial culture should be monitored regularly so that MTB colonies identified can be used directly for PCR-RFLP. The proposed diagnostic algorithm would possibly shorten the turnaround time for identification of INH-resistant MTB associated with katG Ser315Thr substitution. However, this PCR-RFLP method only detected 51% of all INH-resistant MTB in this study. The remaining resistant isolates possibly acquired the INH-resistant phenotype by accumulation of novel mutations in katG or known mutations of other gene loci, such as inhA or abpC (Basso et al., 1998; Lee et al., 2001; Mdluli et al., 1998; Sherman et al., 1996). Further understanding of INH-resistance mechanisms in MTB will facilitate the development of PCR-based protocols for rapid diagnosis of the pathogen in clinical specimens.

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REFERENCES


315 of the *katG* gene are associated with high-level resistance to isoniazid, other drug resistance, and successful transmission of *Mycobacterium tuberculosis* in the Netherlands. *J Infect Dis* 182, 1788–1790.

